Bio-Works

WHITE PAPER

IEX and RPC compatibility in peptide purifications: a generic procedure for implementing IEX upstream of RPC

Peptides as therapeutic molecules

The peptide market is rapidly expanding, and this growth is mainly attributed to increasing investments in therapeutic peptides with the increasing prevalence of infectious diseases and metabolic disorders. The development of COVID-19 vaccines has increased utilization of peptides which in itself will have a positive impact on market growth. Moreover, governments are authorizing extensive support for COVID-19 vaccine development (that includes peptides) and this is also seen as a potential opportunity for adoption of peptides. The main advantages of using peptides as therapeutics compared to more traditional biologics are simpler design, cheaper synthesis, high biological activity, high specificity, and low toxicity. However, there are still significant challenges for the pharmaceutical industry in bringing peptides to market with many adopting greener peptide synthesis techniques at increased costs over traditional approaches. All these factors put demands on the downstream purification processes, in which high purities, high yields and greener processes are the goal.

Crude synthetic peptide feeds (solid phase synthesis and/or liquid phase synthesis) contain impurities such as truncated, deleted, isomerized and deprotected biproducts. Custom-made peptides are routinely synthesized to up to 50-60 amino acids, but the longer the peptide sequence, the more abortive and erroneous sequences must be removed in downstream purification processes. With the addition of every new protected amino acid the overall recovery is reduced. Even with 99% coupling efficiency, the yield for a 40-mer peptide is only about 65%. For a 20-mer peptide, a higher yield of 82% is expected, see Figure 1.

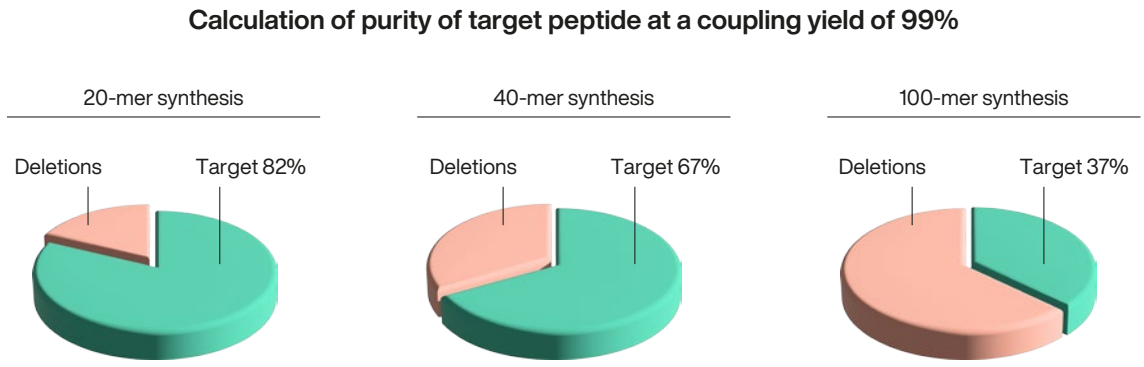


Figure 1. The challenge with synthetic peptides. Diagram of a 20-mer, 40-mer and 100-mer peptide syntheses with a coupling efficiency of 99%.

RPC – The only choice?

The most common way to purify synthesized peptides is using reverse-phase chromatography (RPC), often with C18 silica-based columns. For bigger peptides, such as insulin, C4 or C8 RPC is often preferred. High purities and relatively high yields can generally be obtained using RPC as a stand-alone step. Therefore, RPC is the first choice when screening conditions at lab-scale or analytical scale, but it has some drawback. It has a sensitive silica-backbone that can easily be fouled since it does not tolerate alkali cleaning conditions, for example, hydrophobic impurities derived from the synthesis can accumulate and irreversibly bind tightly to the RPC resin. It is also difficult to scale up without generating very high backpressures. Additionally, it is not an entirely faultless technique, and has issues resolving some impurities and contaminants. This especially applies to polar analytes, which may be poorly resolved on C18.

Orthogonality: separation based on a fundamentally different chromatography principle

To circumvent the problem, one can employ an orthogonal chromatography step upstream of the RPC, using a more hydrophilic and polar resin that tolerates the required harsh cleaning conditions and at the same time resolves the peptides and the impurities to enhance the final purity of the target. Ion exchange chromatography (IEX) fills this criterium and is fully orthogonal to RPC. Where RPC separates molecules based on hydrophobicity, IEX separates molecules based on charge.

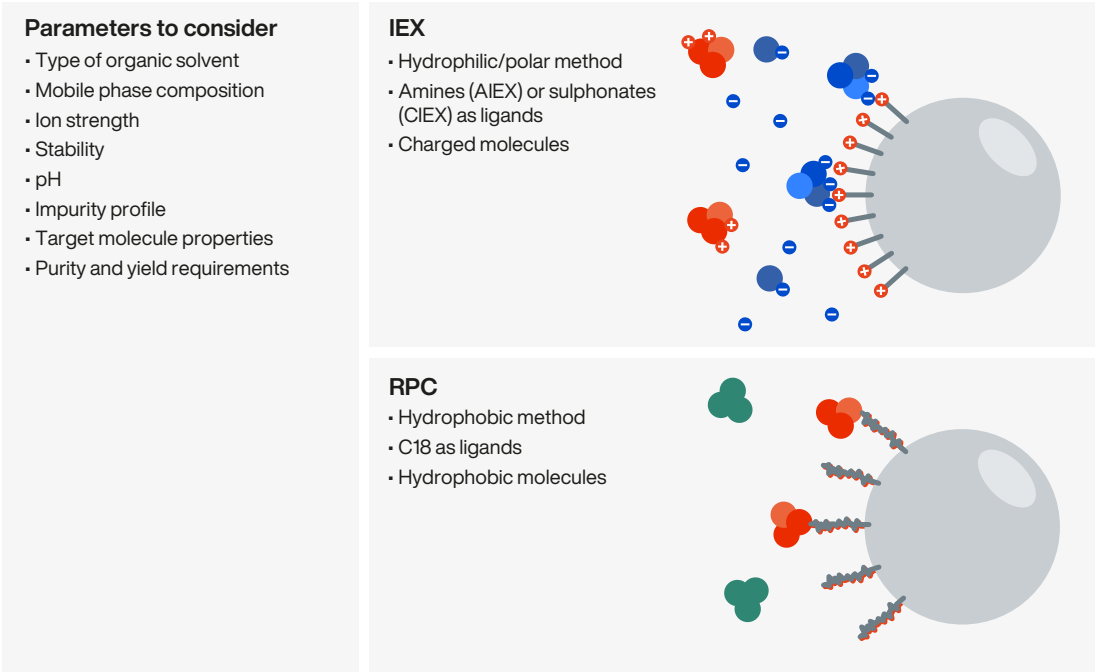


Figure 2. IEX and RPC are two orthogonal chromatographic techniques that separate molecules based on charge and hydrophobicity, respectively.

It has long been recognized that complete resolution of all components of a complex mixture is extremely challenging using a single chromatographic method. Therefore, the use of a generic orthogonal method is needed. In this white paper we described an easily implemented IEX protocol that can be used upstream of RPC and that is directly compatible with RPC without the need for significant changes in buffer systems.

Easy generic IEX method to use: 0.1% TFA

Since the net charge of peptides varies with pH, their net hydrophobicity also varies with pH. The eluent pH has therefore an important influence on elution order and final selectivity. A common practice in RPC is to use a strong acid as binding buffer that also acts as an ion-pairing agent, *i.e.* 0.1% trifluoroacetic acid (TFA), pH 2. An ion-pairing agent is used to enhance the hydrophobic interaction between the analytes and the ligands (*e.g.* C18) and to minimize mixed-mode interactions, such as ionic interactions. If we now use the RPC binding buffer as the IEX binding buffer, we must use a strong cation exchanger (CIEX), since the peptide will be cationic; the amino acids are protonated, at this pH. The advantage of employing such an acidic pH is that even unknown sequences will be bound to the ligand on the chromatography CIEX resin (sulphonate).

0.1% TFA in the mobile phase will not be a proper buffer system, but it will still be good enough to separate most peptide species. The addition of 5-20% acetonitrile (ACN) in the mobile phases will prevent non-specific hydrophobic interactions avoiding mixed-mode interactions, thus enhancing the final orthogonality of the combination of IEX – RPC. Only 1-2 positive charges (histidines, lysines or arginines) in the sequence apart from the amino terminal charge are needed for a peptide to adsorb to the resin, which means that most peptides will bind. Depending on the isoelectric point of the peptide, the pH can be adjusted to achieve optimal retention, especially for more basic peptides which may be difficult to elute if the pH is too low.

- Binding condition (mobile phase A): 0.1% TFA, 5-20% ACN
- Elution condition (mobile phase B): 0.1% TFA, 5-20% ACN, 1 M NaCl

Proof-of-principle

The therapeutic peptide Bivalirudin is a highly effective and selective thrombin inhibitor, making it an effective alternative to heparin as a blood thinner. Bivalirudin is commonly purified using only RPC, but this can be inefficient due to impurities being similar to the target, which results in challenging separations. The target peptide sequence contains two positive charges, arginine and the amino terminal, and it exhibits a relatively low degree of hydrophobicity, which means it will be adsorbed to a CIEX resin at low pH.

To test the feasibility of this proposed generic IEX – RPC purification, we loaded a crude Bivalirudin with a start purity of 85% onto a column packed with WorkBeads™ 40S (Bio-Works) at a flow rate of 150 cm/h, which equals a residence time of four minutes. WorkBeads 40S is a strong, agarose-based CIEX resin with a bead size of 45 µm and a pore size less than 500 Å. A small-scale column was loaded to achieve maximal separation between target peptide and impurities.

Only one symmetrical peak was obtained with no visual separation between peptide species. Most impurities are expected to be eluted in the beginning and at the end of the peak, thus the middle of the peak was collected, with the start and end excluded, see Fig. 3A. The collected pool of 4 mL was analyzed and further loaded onto a semi-preparative RPC column (C18 SPS-10, Chromatorex, bead size 10 µm, pore size 150 Å) (Fig. 3B). The aim of combining these orthogonal techniques, CIEX and RPC, was to achieve a higher final purity of Bivalirudin as compared to purification using either CIEX (Fig. 1A) or RPC as stand-alone steps (Fig. 3C). All collected fractions were analyzed with an analytical RPC (ES-C18 peptides, 2.1 x 150 mm, 2.7 µm bead size, Ascentis).

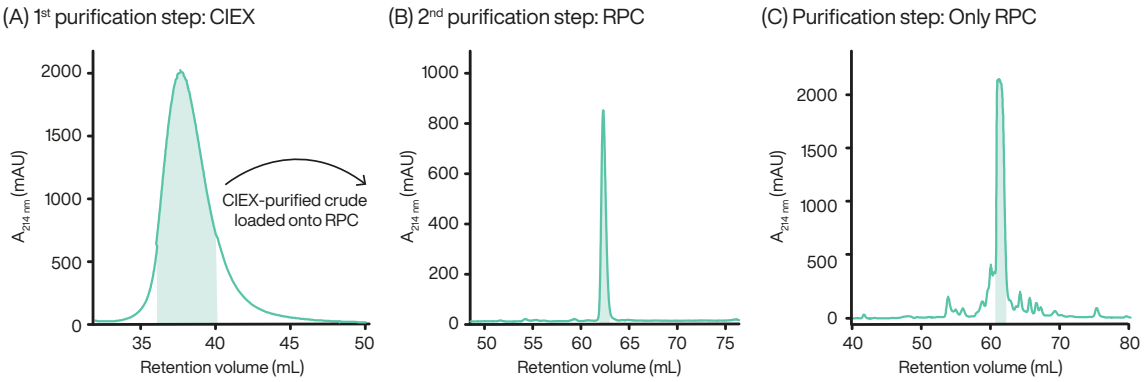


Figure 3. Chromatographic purification profiles for Bivalirudin crude using only CIEX, CIEX-RPC or only RPC. Crude Bivalirudin was loaded onto WorkBeads 40S (A) where the marked fractionation pool was collected and further purified on C18 SPS-10 (B) or the crude Bivalirudin was only loaded onto C18 SPS-10 as a stand-alone purification step (C). UV traces at 215 nm are shown as a solid green line and collected pool volumes as light green areas.

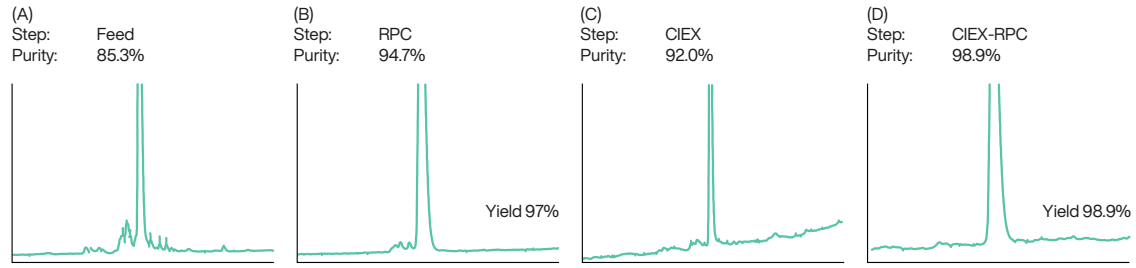
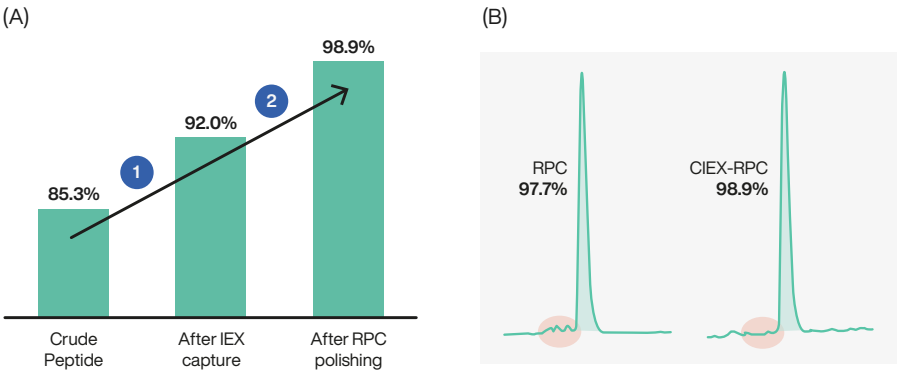


Figure 4. RPC analyses of purified pools from the two-step purification (IEX – RPC) *vs.* one-step (IEX or RPC). Feed (A), eluted pool from C18 SPS-10 alone (B), step 1: eluted pool from WorkBeads 40S (C), step 2: eluted pool: from the combination of WorkBeads 40S and C18 SPS-10 (D).

The analyses of all eluted pools showed RPC was a good stand-alone step in which a purity of 94.7% was obtained at a yield of 97%. However, the combination of CIEX and RPC resulted in a purity of close to 99% at a yield of 90%. See Figure 4 for purity *vs.* yield in the eluted pools from the sub-sequent purification steps. Even though there was no visual separation between the different peptide species in the CIEX purification, the analysis showed significantly improved purity of the crude sample (Fig. 5A).

Interestingly, the early eluting impurities, which were not properly resolved on RPC, were significantly reduced when CIEX was implemented in the process, see Figure 5B.



When combined, IEX and RPC can resolve different kinds of impurities resulting in higher purities.

Figure 5. Purity improvement with CIEX-RPC. The impurity increases with each purification step as indicated in diagram (A). The highlighted area shows the removal of impurity peaks using the setup with CIEX-RPC compared to using RPC alone. Peaks detected by software are light red filled.

Conclusions – Benefit of implementing IEX upstream of RPC

As shown in this White Paper, initial IEX runs are easily set up using RPC-compatible buffers. The complementarity of the two different techniques will facilitate and improve difficult purifications, especially for more polar peptides. Purification setup can be optimized using buffers that are more tailored to IEX, if retention and separation are not good enough. Ammonium acetate is a suitable buffer system, with two pKas of 4.75 and 9.25, so it can be used as a buffer in both CIEX and AIEX and is compatible with RPC.

WorkBeads 40S is easy to scale up and there will be no major increase in COM (cost of manufacturing) at process scale to add an IEX step (USP cost is the major driver with expensive synthesis chemicals, *etc.*) In addition to improvement in purity, the delicate silica-based RPC column will also be protected since IEX, which is used as a clean-up step, uses a resin that is tolerant to harsh cleaning conditions. This will make it possible to run the more expensive RPC resins for a longer time. Finally, it is important to point out that IEX is a greener technology, in which little organic solvents are commonly used.

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Bio-Works, Virdings allé 18, 754 50 Uppsala, Sweden. For local office contact information, visit bio-works.com/contact.

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